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AUTHORITY

BDRL ltr, 13 Sep 1971

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TRANSLATION NO. 12 73

DATE: July 68

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

Bollettino dell Istituto Sieroterapico Milanese, Vol. XXXII, 1953

ACTION OF LISOZYME ON B. ANTHRACIS, by L. Bergamini

The action of Lisozyme on B. anthracis is not well known: according to Fleming and Wolff B. anthracis is affected, be it only in a light way, by lisozyme while Rindello states that this is beyond the activity of such an enzyme. Because of the lack of a systematic study, a series of tests were conducted to clarify this problem, by contemporaneously studying the spontaneous germ lisis in simple physiological solution; during the development of B. anthracis in lisozyme solution in broth plus licozyme and in a filtrate of bacterial suspension affected by the lisozyme, was studied during a second period.

MATERIAL AND TECHNIQUE

The tests were conducted on 7 strains of B. anthracis 5 of which had been kept in a laboratory for some time while one had recently been isolated. The germ suspensions had been made by diluting an agar culture of 24 hour incubation in 5cc of physiological solution; 5cc of this suspension were mixed with an equal quantity of lisozyme (1%) solution (Armour) in physiological solution and for control 5cc of the same suspension were mixed with an equal quantity of physiological suspension. Both suspensions were kept at 37° and at varying intervals: 30 minutes, 1 h, 2 h, 3 h, 5 h, 8 h, 18 h, they were checked for clarity and at the same time slides were prepared which were colored by the gram as the Fraenkel methods to demonstrate germ contents; 3cc of each of the suspensions were poured Ostwald viscosimeters which were maintained at 31° in

a constant temperature both during the entire experiment; the viscosity readings were taken: 2 minutes, 10 minutes, 30 minutes, after the beginning of the experiment, and after this every 30 minutes until the tenth hour; a last reading was taken after the 24th hour.

A Leitz photometer was used to determine the turbidity of the microbial suspensions; the turbidity calculation was made by using the initial reading as 100 and applying the following proportion:

Example: original transmission: 100 = transmission after 30 minutes: x; x - 100 represents the percentual quantity of transmission after 30 minutes and hence the corresponding diminishing of suspension turbidity.

The B. anthracis suspension was rapidly centrifuged to determine the ribonucleinic acid and the germs were again suspended in physiological solution, therefore to eliminate the traces of agar passed into the solution 1cc of 1% lisozyme solution in physiological solution was added to 5cc of bacterial suspension while another 5cc of the same suspension were kept for control; the two suspensions were maintained at 37° for various lengths of time: 1 hour, 2 hours, 5 hours and later were centrifuged. 1cc of lisozyme solution was then also added to the control liquid and the ribonucleinic acid contents was then determined with a Beckman spectrophotometer.

The media for B. anthracis culture were prepared by mixing lisozyme with sterile broth or with physiological solution in various proportions as will be shown in the individual experiments and sterilizing the medium by means of a Seitz filter.

LISIS ACTION OF LISOZYME ON B. ANTHRACIS

By studying the preparates colored by the gram method, it has been possible to establish that while in the control normal gram-positive germs only in slightly shortened rows with a few rare elements of gram-negative could be found after 3 hours of experiment, the suspension containing the lisozyme showed numerous isolated germs after 1 hour of experiment, with rows of 4-5 elements, some of which were gram-negative or gram-positive with two polar granules. Lengthening the experiment to 5-8 hours showed numerous gram-negative some nearly discolored germs in the control, while a strong drop in visible germs was noted in the lisozyme suspension, some having changed to mere shadows, however always conserving a very scant gram-positive and normal element. After 18 hours one could note that the lisis proceeded in the control, even though slowly, while the lisozyme suspension showed reappearance of normal, gram-positive germs, in rows of 4-5 elements. In following experiments, one observed that by using larger lisozyme dilutions (1:5000 - 1:10000) the lisis advanced more slowly, but was always clear and precise. During the germ lisis the spores remained intact both in numbers and color; once the germ lisis completed they could be seen free in the surrounding medium.

The study of the behavior of turbidity of B. anthracis suspension has demonstrated that while the turbidity of the control diminished slowly after the third hour (3% after 3 hours; 5% after 5 hours; 15% after 8 hours; 28% after 18 hours) the turbidity of the lisozyme containing suspension already diminished after the first hours (3% after the first hour; 17% after 3 hours; 23% after 5 hours, after this remaining stable until the 18th hour of the experiment).

The result of the study of viscosity of the B. anthracis suspension showed that even when mixed with lisozyme it showed the same viscosity as

the original physiological solution, and this viscosity showed no modification during the course of the experiment.

From the study of ribonucleinic acid production it was established that while there was no formation of such an acid in the medium after one hour of contact of the germs with the lisozyme, a limited quantity was produced after 3 hours which increased after 5 hours, while even traces could not be found in the control after 5 hours (See Fig. 1).

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Experiments conducted along the same technique, but repeatedly washing the germs in physiological solution, repeatedly centrifuging and dissolving the residue by means of the mechanical action of a pipette, have shown that B. anthracis treated in this manner, even when suspended in simple physiological solution, was subjected to lisis as rapidly as if it had been in contact with lisozyme; the quantity of ribonucleinic acid which was freed in this manner was in fact equal in both suspensions.

B. anthracis culture in broth and lisozyme and in physiological solution plus lisozyme. Cultivating B. anthracis in broth plus lisozyme (lisozyme 1.50000, or such a dilution as to only cause a very slow germ lisis), even after 7-8 transplantings, did neither affect the germ coloring nor its capacity for spore formation; it was only noted that it developed without forming a film on the surface or filaments and flakes in the suspension, but causing a quasi uniform turbidity in the surrounding liquid; as a matter of fact the disappearance of long rows and the presence of numerous isolated elements could be seen with a microscope. By keeping these cultures at room temperature for a long time (20-30-40 days) the germs kept their shape and color much longer than in the control cultures, made in simple broth where the germs spontaneously went against the phenomenons of involution. The broth plus lisozyme conserved its litic capacity even after B. anthracis development.

B. anthracis was later cultivated in a 1:1000 - 1:2000 - 1:10000 solution of lisozyme in physiological solution; however the germ developed normally in long rows of gram-positive elements whether the lisozyme effect was inhibited by the presence of "liquoid" (Roche) in suitable concentration (Bergamini and Ferrari, 2) or by the heat (80° for 1 hour); this was only true for strong

lisozyme concentrations (1:1000 - 1:2000); the 1:10000 lisozyme dilution proved too weak for the germ development.

B. ANTHRACIS LISIS CULTURE

A B. anthracis suspension was rapidly centrifuged and again suspended in physiological solution to eliminate the traces of agar which could have been sufficient for the germ development (as a matter of fact it had been noted that B. anthracis also developed in physiological solution which only contained traces of agar); lisozyme in a porportion of 1:10000 was added to one section of this solution while the remaining suspension was kept for control. Both suspensions were placed at 37° for 4-5 hours after which lisozyme in a proportion of 1:10000 was also added to the control solution and "liquoid" in doses large enough to deactivate the present lisozyme was added to both solutions (1mg of "liquoid" deactivate 0.25mg of lisozyme). Both suspensions were filtered with a Chamberland candle and the obtained liquids were sterily distributed in sterile test tubes. Thereafter, one proceeded with the B. anthracis seed found in these (both the strain used for lisis and different strains), St. aureus, B. prodigiosum, B. typhi, vibrio choleri; every strain was simultaneously placed in a tube containing a filtrate of the control suspension.

Such test showed that germs only developed in lisate (lisozyme lisis) from B. anthracis; however later tests showed that, if the lisis period was prolonged (24 hours), or, if the germs were lengthily washed and centrifuged before the lisis, germ development was also obtained in the filtrate of the control suspension (lisis in simple physiological solution), however this was always inferior to that obtained in the lisozyme lisate simultaneously executed.

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Therefore it is demonstrated that B. anthracis is lisated by lisozyme and the lisis is accompanied by ribonucleinic acid production. Elements of varying sensitivity to lisozyme exist in one and the same culture: more sensitive elements which are rapidly lisated, it is partly because of their disappearance that the long rows typical of the germ are fragmented, and particularly resistant elements to which the successive germ multiplication is probably due. B. anthracis, however, is not a very sensitive germ to lisozyme; a practically total lisis was obtained during our experiments in 5-8 hours with a lisozyme concentration which causes a complete clearing up of a M. lysodeikticus suspension in only 30 minutes.

In the light of modern theories on stratification of the various constituents on the surface of the bacterial cell, one may assume that the exterior strata of mucopolysaccaride is extremely scant in B. anthracis, as in all germs slightly sensitive to lisozyme, which could also explain the easy lisis of the germ in simple physiological solution; hence for enzyme action, only very small quantities should be passed into solution, because of a simultaneously intervening phenomenon of identical polysaccaride depolymerization, medium viscosity modification can not be brought into evidence, as shown by the experiments.

Resulting from the study of B. anthracis development, we know that the germ develops in broth containing small lisozyme quantities (1:50000) by causing a quasi uniform medium turbidity; the lisozyme, in such small quantities, evidently causes lisis of the elements most sensitive to the enzyme and consequently the fragmentation of germ filaments and flakes, while the more resistant elements multiply normally.

The fact that B. anthracis moves more slowly against the phenomena of involution than when it is not in simple broth is worthy of note; this fact may easily be explained if one considers the results of experiments which have demonstrated that B. anthracis (and other germs: St. aureus, B. prodigosum, B. typhi, Vibro cholera) finds sufficient elements for development in simple physiological solution containing the lisis products of the very B. anthracis. Whence should be remembered that in broth containing small lisozyme quantities even elements which are slightly resistant to the enzyme with time, oppose the litic phenomenon which continually causes new lisis products to enter the medium, useful to the metabolism of the non-lisated elements, and consequently slowing the processes of culture involution.

Furthermore it has been demonstrated that lisozyme alone, in the presence of pure NaCl, may be a sufficient substratum for B. anthracis development; however it is necessary that the lisozyme, only component of the base, be a sufficient concentration (1:1000 - 1:2000) and that its litic activity be inhibited; in fact, an integration of such high concentration, would cause lisis of the germs placed in it; a more diluted form is insufficient for the metabolism of the same germs.

This observation and in a particular way the established fact that the products of germ lisis (in this case B. anthracis) may be eliminated sufficiently for development not only of the same germ but also of various germs suggests certain considerations on the complexity of the concept of disease "receptivity" and of germ "tropism" and its various effects among the various infections and perhaps even on one particular infection and the pathogenic flora habitual host of a subject. We have already had occasion to refer to this last fact in connection with the hyaluronidasic activity found in a non-

pathogenic microorganism (Bergamini, 1951).

SUMMARY

B. anthracis shows a sensibility to the lytic action of lysozyme: the lysis is associated to a decrease of turbidity in the bacterial suspension and to a liberation of ribonucleic acid in the medium.

B. anthracis grows in broth containing small amounts of lysozyme (1:50000), in saline containing great amounts of inactivated lysozyme (1:1000 - 1:2000) and in saline containing the split products of B. anthracis treated with lysozyme.